TECHNICAL NOTE

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Big Game Species Identification by Deoxyribonucleic Acid (DNA) Probes

REFERENCE: Blackett, R. S. and Keim, P., **"Big Game Species Identification by Deoxyribonucleic Acid (DNA) Probes,"** *Journal of Forensic Sciences*, JFSCA, Vol. 37, No. 2, March 1992, pp. 590–596.

ABSTRACT: Species identification is important in many big game forensic science cases but cannot always be accomplished because of the lack of adequate techniques. The authors have developed deoxyribonucleic acid (DNA) probes for elk, deer, and antelope by isolating highly repeated satellite sequences. These DNA probes distinguish among deer, elk, and antelope, although not between different species of deer. Because of the high number of sequence copies per genome, these probes are extremely sensitive, requiring less than 10 ng of total genomic DNA. The developmental protocol for these probes is relatively simple and is applicable to many other species.

KEYWORDS: criminalistics, deoxyribonucleic acid (DNA), big game animals, species identification, wildlife, satellite sequences

Wildlife forensic science has not received the technological attention that human forensic science has, and as a result, fewer tools for discrimination are available. Poaching of big game animals is a common criminal act, and it has been estimated that the illegal harvest may at times approach the legal harvest [*1*].

In wildlife forensic science cases, species identification is often essential to the successful prosecution of an offender. At present, species identification is best accomplished by immunological methods. However, these are limited by the cross-reactivity of antisera among different species. For example, elk will react with anti-deer serum, coyote with anti-dog, buffalo with anti-cow, and mountain lion with anti-cat. For many species, such as antelope, javelina, and game birds, there is no commercial source of antisera. Electrophoretic biochemical tests using serum albumin [*2*] or the enzymes phosphoglucose isomerase [*3*] and erythrocyte acid phosphatase [*4*] can add some specificity to the analysis. Often immunological and biochemical tests will only identify evidential samples to the

This work was supported by grants from Northern Arizona University Organized Research and the United States Department of Agriculture (#91-3730-6224). Received for publication 11 March 1991; revised manuscript received 24 June 1991; accepted for publication 24 July 1991.

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level of the family (that is, deer to the level of the Cervidae). Often, trial testimony is no more conclusive than ". . . having similar immunological and biochemical properties to . . .". Big game hair samples are a common type of evidence but are particularly difficult to identify accurately [5]. Trial testimony concerning hair is usually limited to that the hair is "consistent with that of a particular species." Sensitive species-specific techniques need to be developed in this area of forensic science.

Deoxyribonucleic acid (DNA) analysis has proven effective at distinguishing between human individuals [6], and these same "DNA fingerprinting" probes have been successful at identifying individual deer and elk [7]. DNA fingerprinting probes, however, are too variable for species identification. In addition, they require approximately 1 μg of DNA for Southern analysis, which is often more than can be obtained from a small bloodstain or a single hair. If the discrimination required is merely between species, other regions of the genome may be more applicable. For species identification to be successful, diagnostic DNA sequences must be variable between, but not within, species.

Highly repeated satellite sequences have been observed in nearly all eukaryotes and are highly conserved within a species [8]. These are easily observed following restriction digestion because of their very high copy number (frequently between 10^4 and 10^5 per genome) and their tandem arrangement. A restriction site occurring once per repeat will define the monomer repeat size, although ladders of dimers, trimers, and so forth, are often observed because of random mutations. The very high copy number and specific size classes of satellite DNA mean that Southern analysis may be many orders of magnitude more sensitive than comparable single-copy or multilocus DNA fingerprinting probes. In this study, the authors report the development of satellite sequence probes specific for three big game animals and propose their use in evaluation of legal evidence. These probes generally reveal species-specific hybridization patterns and a sensitivity level that allows minute samples to be evaluated.

Material and Methods

Animal tissues were kindly provided by the Arizona Game and Fish Department and were originally collected at hunter's check stations. DNA isolation from approximately 0.2 g of muscle tissue was done according to the method of Mullenbach et al. [9]. This involves a proteinase K digestion, followed by a high salt extraction with chloroform. DNA was concentrated by ethanol precipitation, rinsed in 80% ethanol, and then dissolved in TE [consisting of 10m*M* tris(hydroxymethyl)aminomethane (Tris) at pH 8 and 1m*M* ethylenediaminetetraacetic acid (EDTA)]. Quantification was done using a Hoefer minifluorometer and Hoescht dye No. 33258. The quality of the DNA was determined by agarose electrophoresis. Restriction digestions were done according to Promega specifications. Agarose gel electrophoresis and the Southern transfer procedure were done according to Maniatis et al. [10]. In brief, samples were loaded onto 0.7% agarose gel containing 1× TRIS, acetic acid, EDTA (TAE). Typically, DNA was electrophoretically separated for 16 h with 25 mA of current. DNA was transferred out the gel and onto nylon membranes (MSI) using capillary transfer. The transferred DNA was covalently linked to the membrane using ultraviolet (UV) irradiation (Stratalinker, Stratagene, Inc.). Radioactive DNA probes were developed by excision of specific restriction fragments following electrophoretic separation in low-melting-point agarose gels (LMP agarose, Fisher Scientific). The resulting agarose plug was then used as the template in a random hexamer labeling reaction [11]. DNA hybridization was performed in an aqueous cocktail [0.6*M* sodium chloride (NaCl), 0.12*M* Tris at pH 8, 0.008*M* EDTA, 0.1% saturated sodium pyrophosphate, 0.1% sodium dodecyl sulfate (SDS), 20 μg/mL of denatured salmon sperm DNA, 1× Denhart's solution, and 6% polyethyleneglycol] at 65°C. The membranes were washed at 65°C for 30 min three times in 0.2 × standard saline citrate

(SSC), with 0.5, 0.2, and 0.1% SDS, respectively. The membranes were analyzed by autoradiography or fluorography (with enhancing screens) for various lengths of time, ranging from 30 min to several days. Lambda-phage DNA molecular-weight standards were present on all the electrophoresis gels and were detectable on the hybridization membrane as a result of the addition of radioactive lambda DNA to the hybridization cocktail.

Results and Discussion

Highly repeated satellite sequences can be observed by restriction digestion, followed by electrophoretic separation of DNA fragments. Simply staining the agarose gels with ethidium bromide reveals discrete bands against a more continuous background (Fig. 1). In all, the DNA of six species (elk, mule deer, white-tailed deer, antelope, buffalo, and cow) was examined by digestion with different restriction enzymes (data not shown). All were observed to have highly repeated DNA sequences following digestion with at least one restriction enzyme. Highly repeated DNA sequences were observed in antelope DNA when digested with three different restriction enzymes (Fig. 1). *Eco*RI and *Hin*dIII digestions gave similar patterns, with a major DNA size class band at approximately 1.6 kilobases (kb). A different pattern was observed with *Pst*I digestion where two major DNA size class bands (approximately 1.3 and 0.6 kb) were observed. Buffalo and mule deer DNA both contain a 0.8-kb major DNA size class band when digested with *Pst*I, though these appear to have been different sequences, since hybridization did not occur between them (Fig. 2). *Pst*I-digested elk DNA had the most prominent major size class band, at 1.0 kb. Approximately 20 individuals from each of the three species (elk, mule deer, and antelope) were analyzed by restriction digestion and separation by electrophoresis. Within a species, all the individual animals had identical DNA fragment patterns, as revealed by ethidium bromide staining. The DNA restriction size class bands observed with ethidium bromide staining discriminate between some species but not, for example, between buffalo and mule deer.

In order to develop a species-specific and highly sensitive technique, we used individual DNA size classes as hybridization probes. A discrete DNA size class was isolated from antelope (1.3 kb), elk (1.0 kb), and mule deer (0.8 kb) for use as a radioactive molecular hybridization probe. Genomic DNA in each agarose plug was radioactively labeled and then incubated with a Southern transfer membrane containing DNAs from 10 different species. The hybridization pattern for each probe was unique and distinguished between most species (Fig. 2). The elk 1.0-kb probe hybridized to both elk and deer DNA; however, the *Pst*I restriction pattern for each of these was quite different. Elk DNA contained small fragments (approximately 1 to 2 kb), while with deer DNA, high-molecular-weight (approximately 20 kb) DNA fragments hybridized with this probe. The mule deer 0.8-kb probe hybridized with both elk and deer samples, but again, the hybridization pattern for deer was different from that for elk. Low-molecular-weight fragments (approximately 1 to 2 kb) and some high-molecular-weight fragments hybridized in deer, while primarily high-molecular-weight DNA hybridized in elk. This deer probe hybridized with some relatively faint low-molecular-weight elk fragments, but their sizes were different from those of the deer samples. The antelope 1.3-kb probe hybridized with a series of antelope DNA fragments (approximately 0.5 to 1.5 kb) but not with those of any other species. Further work (not presented) explored the uniformity of the patterns among different individuals within a species. Multiple individual samples of elk, mule deer, and antelope DNA (*Pst*I-digested) were hybridized with these three probes. The within-species patterns were identical.

Forensic discrimination between antelope, deer, and elk was easily accomplished using

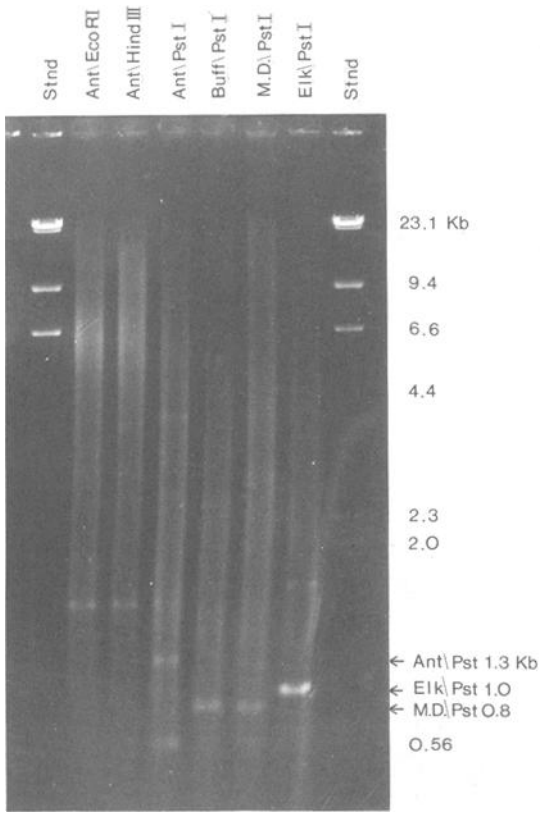

FIG. 1—*Species-specific hybridization patterns of highly repeated DNA probes. DNAs from different species (M.D. = mule deer; Ant = antelope; Buff = buffalo; W.T.D. = white-tailed deer; Jav = javelina; and Stnd = lambda DNA size standard) were digested with* Pst*I and then separated by electrophoresis followed by Southern blotting. Buffalo, elk, mule deer, and antelope are represented by multiple individuals, as indicated. The different panels represent molecular hybridization patterns revealed by the* (A) *Elk* Pst*I 1.0-kb,* (B) *mule deer* Pst*I 0.8-kb, and* (C) *the antelope* Pst*I 1.3-Kb DNA probes (see Fig. 1). Four micrograms of DNA were applied to each lane.*

the techniques developed here. The antelope 1.3-kb probe hybridized only with antelope DNA, making it diagnostic for antelope. Since elk and deer probes hybridize with both species (but not with antelope), the discrimination between deer and elk is dependent upon the unique *Pst*I restriction patterns. Fortunately, these patterns are very different. The deer sequences hybridizing with the elk probe (*Pst*I 1.0 kb) are mostly lacking *Pst*I restriction sites. Thus, the bulk of hybridizing DNA electrophoretically migrates with undigested DNA. Likewise, elk sequences hybridizing with the deer 0.8-kb probe have few *Pst*I sites and primarily migrate at a high molecular weight. Mule deer and white-tailed deer DNAs have identical hybridization and restriction patterns with both the elk and deer probes. Legal distinction between these two species is important but cannot be accomplished using these probes. These two species of deer are very closely related, even to the extent of successful intermating [*12*]. Mitochondrial DNA is a potentially species-specific genetic marker [*12*] between these deer species.

Highly repeated DNA sequences are easily detected in small amounts of genomic DNA. Serial dilutions of *Pst*I-digested mule deer DNA were electrophoretically separated

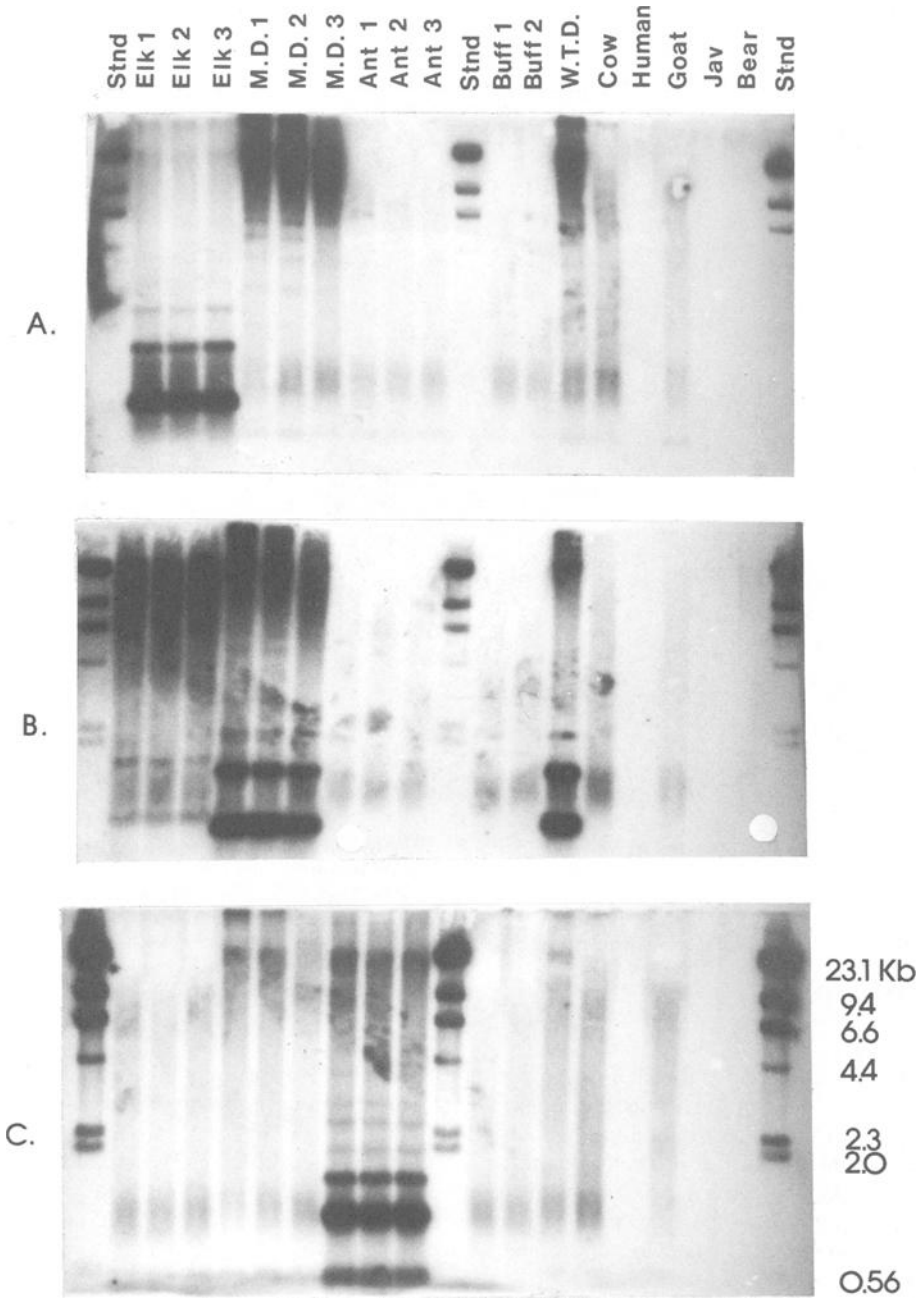

FIG. 2—*Electrophoretic separation of highly repeated DNA fragments. DNA from four species (M.D. = mule deer; Buff = buffalo; Ant = Antelope; and Elk) were digested with the restriction enzymes* PstI, Hind*III or* Eco*RI and then separated according to size by agarose gel electrophoresis. Highly repeated DNA fragments were observed by staining the gel with ethidium bromide. The three fragments indicated by arrows were used as molecular probes in Fig. 2. The fragment sizes of the molecular weight standards (*Hind*III-digested lambda phage) are indicated on the right. Five micrograms of DNA were loaded per lane.*

in order to determine the limit of sensitivity available with these highly repeated DNA probes (Fig. 3). After a two-day exposure, the lowest dilution to have a detectable hybridizing fragment contained 100 pg of DNA. One nanogram of DNA is easily observable with a two-day exposure. The elk and antelope repeat probes were sensitive to the 100 pg and 10 ng levels, respectively (data not presented). Our experience (unpublished data) and that of others [*13*] is that a single hair root contains more than 10 ng of total DNA. Our preliminary analysis of single deer and elk hair samples has successfully identified their source (manuscript in preparation). The sensitivity level observed in Fig. 3 would allow some of the hair DNA to be sent for independent analysis.

The sensitivity level reported in this study should not be considered the maximum level that can be achieved. DNA fragments isolated directly from a total genomic restriction digestion (as reported here) will be enriched for repeated sequences, but are not pure. The lack of purity will decrease sensitivity and could potentially increase the background hybridization. Probe DNA purity can be increased by molecular cloning of satellite sequences, which will eliminate all extraneous sequences. Molecular cloning is also essential to ensure procedural uniformity between laboratories. Molecular cloning of these sequences is currently in progress.

Highly repeated DNA probes could be developed for a variety of species. Tandemly repeated DNA sequences have been widely reported in the literature. We have surveyed approximately twelve different mammalian species and observed repeated DNA sequences in all but one (the javelina, data not presented). The initial screening for discriminating satellite sequences for other species could be accomplished quite simply by the procedures described here. In the future, repeated sequences could be molecularly cloned to provide a uniform source of probe DNA. The sensitivity and species specificity we have reported indicate that repeated DNA probes can be powerful tools in wildlife forensic science and their development merits further research.

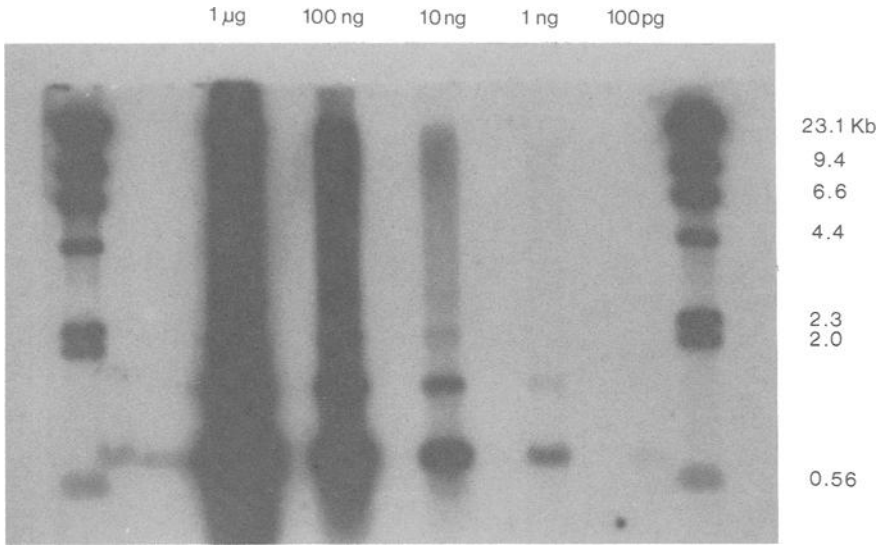

FIG. 3—*Sensitivity of a highly repeated DNA probe.* PstI*-digested mule deer DNA was serially diluted and then separated by electrophoresis followed by Southern transfer. Radioactive M.D.* PstI *0.8-kb DNA was used as a hybridization probe. The amount of total genomic DNA per lane is indicated. The transfer membrane was exposed to X-ray film with enhancing screens at −70°C for two days. The sizes of the lambda molecular weight standards are indicated on the right.*

Acknowledgments

This work was supported by grants from N.A.U. Organized Research and the United States Department of Agriculture to P.K. The authors would like to thank the Arizona Game and Fish Department and Dr. Gary Bateman for furnishing the samples. We are also grateful to James Schupp, Marilee Sellers, and William Donovan for technical assistance.

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